1939

A classification of the proteolytic micrococci isolated from dairy products

Horace Hammerton Harned
Iowa State College

A CLASSIFICATION OF THE PROTEOLYTIC MICROCOCCI ISOLATED FROM DAIRY PRODUCTS

by

Horace H. Harned

A Thesis Submitted to the Graduate Faculty
for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject Dairy Bacteriology

Approved:

Signature was redacted for privacy.

In charge of Major work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1939

UMI Number: DP12737

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

TABLE OF CONTENTS

INTRODUCTION

In studying changes in dairy products, bacteriologists have given the micrococci relatively little attention since it is generally thought that these organisms produce changes slowly and therefore are of little importance. The usual classifications of the micrococci are based on such characteristics as gelatine liquefaction, nitrate reduction and the fermentation of various carbohydrates. These characteristics are of little practical value to the dairy bacteriologist. The action of the organisms on the constituents of milk, however, may be of considerable importance.

Various micrococci found in milk and milk derivatives are capable of attacking the proteins of milk. The extent to which the milk is proteolyzed varies greatly with the particular organism. In some instances proteolysis can be detected by the appearance of the milk while in other instances chemical analyses are necessary to detect changes.

The ability of certain micrococci to break down proteins is important since many dairy products contain enough proteins to make such changes undesirable from the standpoint of quality; however, such changes may be of importance in cheese ripening. The micrococci, therefore, should be seriously considered by the dairy bacteriologist.

STATEMENT OF PROBLEM

The objects of the work herein reported are:

1. To isolate proteolytic micrococci from various dairy products and to study their general characteristics.

2. To determine the effect of various types of proteolytic micrococci on milk and butter.

3. To classify the proteolytic micrococci in dairy products and to identify them.

HISTORICAL

The name Micrococcus was first applied by Hallier (18) to indicate the growth form of a mold and, later, by Cohn (8) to all spherical bacteria. In 1872, Cohn (8) published his conception of the systematic relationships of bacteria and for the first time the coccus types were placed in a separate tribe on an equal basis with other tribes of bacteria. The genus name Micrococcus was used for this group which included all spherical bacteria; a definite description of the genus was given. In 1875, Cohn (10) proposed a more complete classification of bacteria than anyone else had attempted up to this time. His classification was based on the assumption that the various types were degenerates of the blue-green algae. Having in mind the close relationship between the algae and the bacteria, he arranged those species of bacteria which he supposed were closely related to species of blue-green algae in the same genus with the algae so that many genera contained both bacteria and algae. The streptococci and sarcina were distinguished by their respective chain and packet formations.

The genus Micrococcus of Migula (31) and of Chester (7) was a kind of residual genus including all cocci which do not occur in well marked chains or packets. One of the outstanding characteristics of the group is the ability to produce

pigments, and many investigators have used this character as a starting point for classification.

In 1908, Dudgeon (12) studied the relationships of a group of staphylococci with particular emphasis on the use of chromogensis for separating the individual species. Working with 46 strains of the orange type and 71 strains of the white type, he observed their liquefying power, their reducing power and their acid producing power in 11 carbohydrate media. His conclusion was that all the staphylococci studied were members of one species, although only a few of the strains were identical in all characters studied.

The Winslows (35), in 1908, made a comprehensive study of the coccus group. A large number of cultures was studied and divisions were made on the basis of habitat, chromogenesis and cell division. They divided the family Coccaceae into two sub-families, the Paracoccaceae which included the parasitic types and the Metacoccaceae which included the non-parasitic or free living forms. The Paracoccaceae were further divided into five genera on the basis of cell division and pigment production. The old genus Staphylococcus was divided into two new genera, namely, Aurococcus which included the orange pigment producers, and Albococcus which included the porcelain white or non-pigment producers. The Metacoccaceae were divided into three genera, principally on the basis of cell formation and pigment production.

Orla-Jenson (32), in 1909, proposed a classification of

bacteria based primarily on physiological characters, the most important of which was acid production in various carbohydrate media. Although the conventional morphological grouping of the cocci was recognized, only the physiological characteristics were used in his classification. His classification of the coccus group is as follows:

Family Acidobacteriaceae
Genus Streptococcus
Genus Micrococcus
Genus Sarcina

In 1917, Buchanan (5) classified the micrococci on the basis of their physiology and habitat. The genera were separated on the basis of cell division, habitat, and chromogensis. The tribe Micrococceae was divided into three genera:

Genus Micrococcus
Genus Rhodococcus
Genus Sarcina

Using the cultures of the American Museum of Natural History Collection, Kligler (26), in 1913, confirmed the work of the Winslows (35) and concluded that the white and orange micrococci were usually parasitic strains while the yellow and red micrococci were more often saprophytic.

In 1920, Winslow and his associates (36), after further study of the micrococci, were doubtful of the validity of the genus Aurococcus and genus Albococcus, described in 1908. Since the organisms in the two genera were similar, these investigators combined the groups into the single genus Staphylococcus.

Hucker (24) reported an exhaustive study of the micrococci and was convinced that chromogenesis of the members of this group is a very important character for use in classification but that groups should not be divided into genera on the basis of chromogenesis as the chief diagnostic feature. A study of the constancy of various characters of this group showed that chromogenesis, nitrate reduction, ability to utilize ammonium salts as the only source of nitrogen, gelatine liquefaction, and action on milk are tests whereby the natural species of the group can be separated.

In 1920, the committee on Classification of Bacterial Types, appointed by the Society of American Bacteriologists, (37), arranged the Micrococceae into three genera:

Genus Micrococcus
Genus Sarcina
Genus Rhodococcus

This is similar to Buchanan's classification (5).

Bergey (5), in his Manual of Determinative Bacteriology, divided the Micrococceae into five genera:

Genus Staphylococcus
Genus Gaffkya
Genus Micrococcus
Genus Sarcina
Genus Rhodococcus

For over 50 years research workers in the field of dairy industry have known that micrococci were plentiful in dairy products. Gorini (17), in 1901, reported micrococci as the most common type of organism in fresh milk. He thought the proteolytic micrococci in the mammarian glands were advan-

tageous in the digesting of milk and in the ripening of cheeses. In hard cheeses the organisms reduce casein to more digestible forms during ripening and at the same time promote the development of lactic acid organisms which contribute to flavor development. Liquefying, chromogenic micrococci were found by von Freudenreich (16) to predominate in milk. Orla-Jensen (32) reported many micrococci and sarcina in milk and termed them tetracocci. The sources of the tetracocci were cow manure, soil, dust, the coat of the animal and the skin of the milker. According to Orla-Jensen, the organisms play an important part in the ripening of cheeses.

In an extensive study on the microflora of milk, Harding and Wilson (21) found that 75 per cent of the organisms present in freshly drawn milk were micrococci. In a similar study, Evans (14) noted that the predominant flora in fresh cows' milk consisted of Gram positive micrococci. Copeland and Olson (11) found mostly Gram positive micrococci in aseptically drawn milk, some of which liquefied gelatine. Breed (4) reported that the predominant flora in milk consisted of Gram positive micrococci, while Knowles (26) found that 48 of 68 cultures isolated from freshly drawn foremilk were micrococci.

Hucker (23), in examining microscopic sections of old cheddar cheese, found that micrococci predominated. The

characteristic clumping of this group of organisms showed that in their growth and reproduction they probably broke down the surrounding medium for a source of energy. Frazier and Rupp (15) reported the micrococci in milk as mostly acido-proteolytic.

METHODS

Detection of Proteolytic Micrococci

Milk agar plates

Proteolytic micrococci were detected on agar plates by the addition of 5 ml. of sterile skimmilk to 100 ml. of beef infusion agar at the time of plating.

Gelatine liquefaction

Tubes of standard nutrient gelatine were inoculated and incubated at 37°C. for 5 days. The cultures were then placed in a refrigerator at 10°C. for 4 hours. After the holding period, the tubes were examined for liquefaction. Tests were also made by incubating the tubes at 15° to 18°C. for 15 days.

General Characteristics

Action on milk

The action of proteolytic micrococci on milk was determined by inoculating tubes of litmus milk with the organisms. The tubes were examined after 48 hours at 37°C.

pH and titratable acidity

The pH values were obtained electrometrically with the quinhydrone electrode. The titratable acidity of milk cul-

tures was obtained by titrating 20 ml. portions with N/10 sodium hydroxide, using phenolphthalein. The results were calculated as lactic acid.

Nitrate reduction

Nitrate reduction was determined by a method adopted by the committee on technique of the Society of American Bacteriologists (23).

Nitrogen assimilation and ammonia production

Tests for the assimilation of amino and ammonia nitrogen and the production of ammonia from peptone and nitrates were those described by Thaker (24).

Carbon dioxide

The production of carbon dioxide was measured by means of Aldredge tubes (13).

Hemolysis

Hemolysis was studied by streaking cultures on blood agar plates. The plates were prepared by adding 4 ml. of the following mixture to 100 ml. of beef infusion agar: 2 ml. aseptically drawn cow's blood and 2 ml. 5 per cent aqueous sodium citrate solution

Lipolysis

Lipolysis was investigated by the modified nile blue sulfate and natural fat techniques as suggested by Long (29).

Diastatic action

Diastatic action was determined by the method suggested by Allen (1).

Carbohydrate fermentation

The fermentation of carbohydrates was studied by growing organisms in broths containing various materials and also bromcresol purple as an indicator.

Thermal death point

The thermal death point of organisms was determined by heating cotton stoppered tubes containing broth cultures for 15 minutes at various temperatures in a water bath.

Acetylmethylcarbinol plus diacetyl

Acetylmethylcarbinol plus diacetyl was determined qualitatively by the Voges-Proskauer reaction (55) and quantitatively by the procedure employed by Michaelian and Hammer (30).

Volatile acidity

Volatile acidity was determined by the method used by

Michaelian and Hammer (30).

Hydrogen sulfide

Hydrogen sulfide was determined with the method used by Bailey and Lacy (2).

Indol

The production of indol was determined by the Ehrlich-Bohme method adopted by the committee on bacteriological technique of the Society of American Bacteriologists (33).

Pathogenicity

Pathogenicity was tested by inoculating guinea pigs.

Protein Breakdown in Skimmilk

The total soluble nitrogen produced by cultures of proteolytic micrococci was obtained by precipitation of milk cultures of the organisms with acetic acid and then filtering through paper. The nitrogen in both the filtrates and the precipitates were determined by the Kjeldahl method. Amino nitrogen was obtained with the Van Slyke gasometric method (34). Nitrogen soluble and insoluble in trichloracetic acid, phosphotungstic acid and ethyl alcohol was fractioned according to the procedure used by Lane and Hammer (28).

General Action of Proteolytic Micrococci on Butter

Churnings were made from sterile cream inoculated with milk cultures of the proteolytic micrococci. The butter was examined organoleptically at various periods during the holding.

EXPERIMENTAL

Isolation of Proteolytic Micrococci from Dairy Products

Proteolytic micrococci were isolated from milk, butter, cheese (cheddar) and ice cream. The sources of the samples of these materials and the numbers from each are as follows:

SOURCES	Number of samples examined			
	Milk	Butter	Cheese	Ice Cream
Market Milk Department Iowa State College Ames, Iowa	186			
Dairy Department Mississippi State College State College, Mississippi:				
Barn No. 1	148			
Barn No. 2	164			
Dairy of H. H. Harned State College, Mississippi:	82			
A & M Cooperative Creamery: State College, Mississippi:		42		46
Grocery Stores Starkville, Mississippi			36	

All samples of milk were taken from fresh morning milk; each sample (about 10 ml.) was collected in a sterile test tube. The samples of butter were cut from one-pound prints with sterile spatulas and held in petri dishes. The samples of cheese were obtained with sterile triers and held in petri dishes. The samples of ice cream were obtained with sterile

spatulas, and allowed to melt in petri dishes at room temperature.

The samples of the four dairy products were plated with beef infusion milk agar in the following manner: The milk and ice cream were diluted with water as usual. The butter was melted at 45°C. and dilutions were made with warm water. The cheese were ground in a mortar with 10 ml. of 2 per cent aqueous sodium citrate until a homogeneous liquid was obtained; the necessary dilutions were made with water. All plates were incubated at 37°C. for 48 to 72 hours. Those colonies which were smooth and round, and which were encircled with a clear area characteristic of proteolytic organisms, were streaked on beef infusion milk agar plates for purification and for identification as micrococci. Special care was exercised to avoid duplicating organisms from a sample. In order to determine the morphological characters, smears were made and stained. After an organism had been identified as a Micrococcus by microscopic examination, it was transferred to an agar slant as a stock culture for detailed study. From 704 samples of dairy products, 426 proteolytic micrococci were isolated. The general sources of the organisms are shown in the following summary:

	Milk	Butter	Cheese	Ice Cream
Number of samples examined:	560	42	36	46
Number of samples yielding proteolytic micrococci	340	36	20	30

The sources of the individual cultures that were studied in detail are given in Table 1.

Table 1.

Sources of the proteolytic micrococci

Source	Total cultures	Culture numbers
Milk	340	All numbers not listed under butter, cheese and ice cream.
Butter	36	21, 36, 44, 49, 58, 132, 133, 134, 135, 178, 179, 180, 181, 210, 211, 212, 213, 214, 215, 250, 251, 252, 253, 254, 255, 256, 257, 329, 330, 331, 332, 354, 355, 356, 357.
Cheese	20	6, 96, 97, 98, 99, 100, 101, 126, 127, 174, 175, 176, 177, 243, 244, 245, 246, 247, 248, 249.
Ice Cream	30	27, 54, 55, 56, 57, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 169, 170, 171, 172, 173, 333, 335, 324, 325, 326, 327, 328, 341.

Chromogenesis

The pigments produced by the proteolytic micrococci encountered were red, orange, yellow or white (non-pigmented). In this investigation the red micrococci were omitted since only a few were isolated and these grew very slowly at both room temperature and 37°C. The slow development of the red organisms may account for the fact that so few were isolated as most of the colonies were picked from plates that were incubated not more than 72 hours. The cultures studied included 53 orange micrococci, 115 yellow and 358 white. On beef infusion agar, the cultures were very consistent in their pigment production.

Influence of Various Factors on Chromogenesis

Carbon dioxide

Orange cultures 4, 7, 12, 13, 15, 19, 20, 22, 30, 31, 36, 37, 39, 49, 53, and yellow cultures 59, 61, 64, 69, 72, 74, 81, 88, 91, 93, 94, 95, and 97 were streaked on beef infusion agar plates. The plates were placed in a Novy jar and carbon dioxide was allowed to flow into the jar until the air was largely displaced. After 5 and 10 days incubation periods at room temperature the cultures were compared with duplicate plates streaked at the same time but incubated under aerobic conditions. With cultures 7, 13, 15, 31, 36, and 49 held under carbon dioxide, the orange

pigment slightly lighter in color than in the controls, but most of the cultures appeared to produce as much pigment under carbon dioxide as in air. No change in the pigment production was noted with the yellow cultures.

Growth on potato

Orange cultures 4, 7, 12, 13, 15, 19, 20, 22, 30, 31, 36, 37, 39, 49, and 53 and yellow cultures 59, 61, 64, 69, 72, 74, 81, 88, 91, 92, 94, 95, and 97 were streaked on potato to compare the growth with that on beef infusion agar slants. The cultures were held at room temperature. Cultures 7, 13, 36, and 49 showed more pigmentation on potato than on beef infusion agar, while cultures 31, 39, and 47 appeared to have a deeper color on beef infusion agar than on potato. The yellow cultures showed about the same pigmentation on the two media.

pH of the medium

The pH of the medium appeared to affect the amount of pigment produced. The test organisms employed were those used in studying the growth on potato. When agars having values of 6.6, 7.0 and 7.6, respectively, were employed and the cultures held at room temperature, pigmentation was most pronounced on the agar having a pH of 7.0 with all cultures studied but was definite in all cultures.

Incubation in water

Orange cultures 12, 13, 15, 19, 20, 22, 30, 31, 36 and 49, and yellow cultures 59, 61, 64, 69, 72, 74, 81, 93, 97 and 103 were each placed in a tube of sterile water for 6 days at room temperature and then streaked on agar slants. The cultures were incubated at both 30° and 37°C. for 48 hours and then compared with cultures not placed in water. No difference in pigmentation was noted between the two types of cultures.

Heating at 55°C.

Orange cultures 12, 13, 15, 19, 20, 22, 30, 31, 39 and 49, and yellow cultures 59, 61, 64, 69, 72, 74, 81, 93, 97 and 103 were diluted in tubes of water and placed in a water bath at 55°C. for 10 minutes. The cultures were streaked on beef infusion agar and held at 37°C. for 48 hours. With orange cultures 39 and 49 there appeared to be a deeper color produced by the heated cultures than by unheated control cultures, but in the majority of comparisons there was no noticeable change due to heating. No change in pigmentation was noted with the yellow cultures that were heated.

Incubation temperature

Agar plates streaked with orange cultures 4, 7, 12, 13, 15, 19, 20, 22, 30, 31, 36, 37, 39, 49 and 53 and with

yellow cultures 59, 61, 64, 69, 72, 74, 81, 86, 91, 93, 94, 95, and 97 were incubated at 21°C. and also at 37°C. The orange cultures showed a deeper pigment production at 21°C. than at 37°C., whereas the yellow cultures showed no variation with the incubation temperatures.

Darkness

The orange and yellow cultures used in studying the effect of incubation temperature were streaked on duplicate tubes of agar. One set of tubes was placed in closed mailing tubes, while the other set was held in a room having sunlight most of the day. After 10 days the orange cultures incubated in darkness were much lighter in color than those incubated in the light room. With the yellow cultures little difference in pigmentation was noted, regardless of the amount of light present.

Chromogenesis as a Means of Separating Genera

Many workers have made use of chromogenesis as a diagnostic feature in separating genera. Usually it is an easy character to determine and the colors are relatively constant. However, orange culture 58 changed from orange to white on agar but later the orange color reappeared; this color change occurred twice over a 2-year period. In litmus milk cultures an orange ring regularly formed at the surface while agar

streak cultures were white with an orange periphery.

Since the chromogenic properties of the proteolytic micrococci were relatively constant, chromogenesis is apparently a satisfactory means of dividing relatively large numbers of organisms into groups. In this study the chromogenic properties were employed to divide the organisms into the orange, yellow and white groups.

General Growth and Viability of Proteolytic Micrococci

All of the 426 cultures of proteolytic micrococci studied grew well on beef infusion milk agar, although the amount of growth varied considerably among individual cultures.

In general, the cultures were viable for relatively long periods. Litmus milk cultures held 3 to 4 months and agar slant cultures held 10 to 12 months at room temperature remained viable. Agar slant cultures held for long periods were easily revived by adding a small amount of beef broth to the cultures, holding over night and then streaking on agar.

General Characteristics of Proteolytic Micrococci

The general characteristics, grouping and identification of the organisms studied are given in Table 2. The

General characteristics grouping and

Culture no.	Form	Size microns	Gram stain	Action on milk 37°C. 48 hrs.	Titratable acidity in milk 37°C. 48 hrs.	Growth on agar slants	Growth in beef broth	Nitrate reduction	Amino N. assimilation	Ammonia N. assimilation
1 to 48	singly pairs clusters	0.6 to 1.0 av. 0.8	+	acid coag.[2] dig.[3]	0.30 to 0.36 av. 0.33	abundant	turbid	+	-	-
49 and 50	clusters	0.8 to 1.4 av. 1.1	+	inert	0.24 to 0.28 av. 0.26	abundant	turbid	-	-	+
51	singly clusters	0.9	var.[1]	acid coag. dig.	0.39	abundant	turbid	-	-	-
52 and 53	singly fours	0.6 to 1.0 av. 0.8	+	acid	0.22 to 0.26 av. 0.24	abundant	turbid	+	-	+
54 to 95	singly pairs clusters	1.0 to 1.6 av. 1.3	var.	acid coag. dig.	0.1 to 0.20 av. 0.18	abundant	turbid	-	+	+
96 to 113	singly pairs clusters	1.0 to 1.4 av. 1.2	var.	inert	0.14 to 0.16 av. 0.15	abundant	turbid	-	-	+
114 to 137	singly pairs clusters	0.8 to 1.2 av. 1.0	var.	acid coag. dig.	0.14 to 0.18 av. 0.16	abundant	turbid	+	-	-
138 to 168	singly clusters	0.9 to 1.3 av. 1.2	var.	inert	0.12 to 0.14 av. 0.13	abundant	turbid	+	-	-

1 = variable 2 = coagulation 3 = digestion

Table 2.

rouping and identification of proteolytic micrococci isolated from dairy products

Reduction	Amino N. assimilation	Ammonia N. assimilation	Ammonia from peptone	Ammonia from nitrates	Gelatine liquefaction	Carbon dioxide equiv. 0.1 N. acid per 10 ml. milk	Hemolysis	Lipolysis	Diastatic action	Arabinose	Dextrine	Dextrose	Galactose	Glycerol	Inulin	Lactose	Levulose	Maltose	Mannitol	Raffinose
Orange group																				
-	-	-	+	-	saccate	2.8 to 3.2 av. 3.0	+	+	-	-	-	+	+	+	-	+	+	-	+	-
-	+	+	-	strati-form	2.8 to 3.4 av. 3.1	-	+	-	-	-	+	+	-	-	+	+	-	-	-
-	-	+	-	strati-form	6.2	+	+	+	-	+	+	+	+	-	+	+	+	+	-
-	+	+	-	napiform	8.0 to 8.4 av. 8.2	-	+	-	-	-	+	+	+	-	+	+	+	+	-
Yellow group																				
+	+	+	-	strati-form	10.0 to 10.8 av. 10.4	-	-	+	-	-	+	+	-	-	+	+	-	-	-
-	+	+	-	infundibuliform	1.0 to 1.4 av. 1.2	+	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	+	-	strati-form	9.0 to 9.4 av. 9.2	-	-	+	-	-	+	+	-	+	+	+	-	-	-
-	-	+	-	strati-form	5.4 to 6.0 av. 5.7	-	-	+	-	-	+	+	-	+	+	+	+	-	-

i from dairy products

Glycerol	Inulin	Lactose	Levulose	Maltose	Mannitol	Raffinose	Salicin	Sorbitol	Sucrose	Xylose	ermal th int . for 5 minutes)	Classification
+	-	+	+	-	+	-	-	-	+	-	65	Micrococcus aureus
-	-	+	+	-	-	-	-	-	-	-	60	Micrococcus v
+	-	+	+	+	+	-	+	+	+	-	60	Micrococcus w
+	-	+	+	+	+	-	+	+	+	-	60	Micrococcus x
-	-	+	+	-	-	-	-	-	-	-	65	Micrococcus flavus
-	-	-	-	-	-	-	-	-	-	-	65	Micrococcus flavescens
-	+	+	+	-	-	-	-	-	-	-	65	Micrococcus citreus
-	+	+	+	+	-	-	-	-	+	-	65	Micrococcus conglomeratus

Culture no.	Form	Size microns	Gram Stain	Action on milk 37°C. 48 hrs.	Titratable acidity in milk 37°C. 48 hrs.	Growth on agar slants	Growth in beef broth	Nitrate reduction	Amino N. assimilation	Ammonic N. assimilation
169 to 224	singly pairs clusters	1.0 to 1.4 av. 1.2	+	acid coag. dig.	2.20 to 2.8 av. 2.50	abundant	turbid	+	-	-
225 to 241	singly clusters	0.7 to 1.0 av. 0.9	+	acid coag. dig.	0.16 to 0.20 av. 0.18	moderate	turbid	+	-	-
242 to 320	singly pairs clusters	0.6 to 1.0 av. 0.8	+	acid coag. dig.	0.60 to 0.64 av. 0.62	slight	turbid	-	-	-
321 to 405	singly pairs clusters	0.6 to 1.2 av. 0.9	+	acid coag. dig.	0.60 to 0.62 av. 0.61	abundant	turbid	+	-	-
406 to 417	singly pairs clusters	1.0 to 1.6 av. 1.3	+	inert	0.12 to 0.18 av. 0.15	slight	turbid	+	-	-
418 to 424	singly fours	1.5 to 2.0 av. 1.8	+	alkaline	0.12 to 0.16 av. 0.14	moderate	turbid	-	-	-
425 and 426	singly clusters	1.0 to 1.4 av. 1.2	+	alkaline	0.12 to 0.14 av. 0.13	slight	turbid	-	-	-

Table 2. (continued)

Nitrate reduction	Amino N. assimilation	Ammonia N. assimilation	Ammonia from peptone	Ammonia from nitrates	Gelatine liquefaction	Carbon dioxide equiv. 0.1 N. acid per 10 ml. milk	Hemolysis	Lipolysis	Diastatic action	Arabinose	Dextrine	Dextrose	Galactose	Glycerol	Inulin	Lactose	Levulose	Maltose	Mannitol	Raffinose
White group																				
+	-	-	+	-	crateriform	2.8 to 3.2 av. 3.0	-	-	-	-	+	+	+	-	-	+	+	+	-	-
+	-	-	+	-	crateriform	6.2 to 6.8 av. 6.5	-	-	-	-	-	+	-	-	-	+	+	-	-	-
-	-	-	+	-	striatiform	4.0 to 4.8 av. 4.4	-	-	-	-	+	+	+	+	-	+	+	+	+	-
+	-	-	+	-	striatiform	3.0 to 3.4 av. 3.2	-	-	-	-	+	+	+	+	-	+	+	+	-	-
+	-	-	+	-	striatiform	0.1 to 0.3 av. 0.2	-	-	-	-	+	+	+	+	+	+	+	+	-	-
+	-	-	+	-	striatiform	6.4 to 6.8 av. 6.6	-	-	-	-	+	+	-	+	-	-	+	-	-	-
+	-	-	+	-			-	-	-	-	-	-	-	-	-	-	+	-	-	-

Galactose	Glycerol	Inulin	Lactose	Levulose	Maltose	Mannitol	Raffinose	Salicin	Sorbitol	Sucrose	Xylose	Thermal death point (°C. for 15 minutes)	Classification
+	-	-	+	+	+	-	-	-	-	+	-	65	Micrococcus albus
-	-	-	+	+	-	-	-	-	-	-	-	55	Micrococcus cremoris-viscosi
+	+	-	+	+	+	+	-	+	+	+	-	55	Micrococcus freudenreichii
+	+	-	+	+	+	-	-	-	-	+	-	60	Micrococcus caseolyticus
+	+	+	+	+	+	-	-	-	-	+	+	65	Micrococcus epidermidis
-	+	-	-	+	+	-	-	-	-	-	+	55	Micrococcus y
-	-	-	-	+	-	-	-	-	-	-	+	55	Micrococcus z

data show the following relationships:

Action on litmus milk

Changes in litmus milk inoculated with proteolytic micrococci were of five general types:

1. No noticeable change other than an accumulation of cells at the bottom of tubes. This condition was most common with organisms of the yellow group.

2. Producing acid reaction without coagulation. This change was produced by organisms in all groups.

3. Producing alkaline reaction with digestion of protein. The change was produced only by organisms of the yellow and white groups.

4. Producing acid with coagulation and slight protein digestion. The change was noted with all groups of organisms.

5. Producing acid with coagulation and considerable protein digestion. This condition was most commonly produced by members of the orange group.

Although all the cultures were definitely proteolytic on milk agar, the change produced in the appearance of litmus milk by certain of them did not suggest proteolysis.

Titratable acidity produced in skimmilk

With 20 representative cultures titratable acidities varied from 0.12 to 0.66 per cent, calculated as lactic acid. In general, relatively high acidities were produced by members of the orange and white groups while comparatively low acidities were obtained with members of the yellow group. With a few white cultures, however, the

acidities were very low.

Growth on agar slants

All of the cultures studied grew well on beef infusion agar slants. In general, the yellow organisms grew more luxuriantly than those of the orange or white groups.

Growth in beef broth

The organisms regularly produced turbidity in beef broth with the formation of sediment.

Nitrate reduction

A majority of cultures studied reduced nitrates. With the orange group, 50 of 53 cultures were positive; with the yellow group 55 of 115 were positive; and with the white group, 168 of 258 were positive.

Nitrogen assimilation and ammonia production

The assimilation of amino nitrogen was negative with all but a few organisms of the yellow group. Ammonia nitrogen was assimilated by many organisms of the orange and yellow groups; with the white group, the results were negative in all trials.

All of the organisms produced ammonia from peptone, whereas none were capable of producing ammonia from nitrates.

Gelatine liquefaction

All of the cultures liquefied gelatine with the exception of a few in the white group. In general, liquefaction was more rapid and extensive with members of the orange groups than with members of the yellow or white groups.

Carbon dioxide production

Members of the yellow group generally produced larger amounts of carbon dioxide than members of either the orange or white groups.

Hemolysis

Hemolysis was shown by 49 cultures of the orange group and 17 cultures of the yellow group; in the white group, none were hemolytic.

Lipolysis

Lipolytic action by the organisms was limited to the orange group in which all of the cultures gave positive results. Apparently none of the yellow or white organisms studied were lipolytic according to the methods employed.

Diastatic action

The production of diastase was exhibited by 1 culture in the orange group and 96 cultures in the yellow group.

None of the organisms in the white group gave positive results.

Carbohydrate fermentation

In general, most of the cultures studied fermented dextrose, galactose, lactose, levulose and maltose. The fermentation of dextrine and xylose was confined principally to members of the white group, while glycerol, mannitol salicin, sorbitol and sucrose were fermented by some cultures of the orange and white groups. Arabinose and raffinose were not fermented.

Thermal death point

In determining the thermal death points of the organisms, cotton stoppered tubes containing broth cultures were suspended in a water bath at 55°, 60°, 65°, and 70°C. for 15 minutes. All of the organisms were killed at temperatures ranging from 55° to 65°C.

Acetylmethylcarbinol plus diacetyl, volatile acidity, hydrogen sulfide and indol production

Acetylmethylcarbinol plus diacetyl was not produced from milk, with or without added citric acid, by any of the cultures.

Appreciable amounts of volatile acid were not produced by the organisms. Tests for hydrogen sulfide and

indol were negative in all instances.

Pathogenicity, spore formation and motility

To determine the pathogenicity of 30 representative cultures of proteolytic micrococci, the organisms were grown in lactose broth and 2 ml. of a culture was inoculated intraperitoneally into a guinea pig. At the same time guinea pigs were inoculated with cultures classified as Staphylococcus by the Bacteriology Department of the Ohio State University. The animals inoculated with proteolytic micrococci were not affected, whereas several of the cultures from Ohio produced death in 12 to 24 hours.

None of the proteolytic micrococci produced spores and all of the organisms were apparently non-motile.

Protein Breakdown in Skimmilk by Proteolytic Micrococci

Four representative cultures of proteolytic micrococci were selected for the study of protein breakdown in skimmilk. These were cultures 39 (orange), 98 and 110 (yellow) and 170 (white).

Skimmilk, in approximately 200 ml. portions, was sterilized in pint bottles after the weight of each bottle and its contents had been recorded. Several bottles were then inoculated with each culture to be studied and incubated at 37°C. or 31°C. After 2, 4 and 7 day incubation

periods, the serum from each culture was analysed. The serum from a culture was obtained by adding distilled water to make up to the original weight, adding 3 ml. of glacial acetic acid and heating in a bath of boiling water with frequent agitation. After cooling, the culture was filtered through paper. The following determinations were made on 5 ml. portions of the serum: Total nitrogen, amino nitrogen, and the nitrogen soluble and insoluble in trichloracetic acid, ethyl alcohol and phosphotungstic acid. The values were expressed as milliliters of N/10 acid equivalent to the nitrogen in 5 ml. of serum.

Three trials were carried out with the four organisms. The data for one of the trials are presented in Table 3; the data for the other trials are not given since they are practically identical with those given in Table 5.

All cultures of the test organisms produced protein breakdown in skimmilk although the extent of proteolysis varied considerably with the individual cultures. At 37°C., the total nitrogen in the serum of skimmilk inoculated with the organisms increased with continued holding. After 7 days, the values with some cultures were relatively high, especially with culture 170. At 21°C., the values for total nitrogen also increased with continued holding and in some instances the increases were greater than at 37°C. Although there were irregularities with the different incubation periods, in general, cultures 170 and 98 produced

Table 3.

Protein breakdown in skimmilk by certain proteolytic micrococci

Culture no.	Incubation time (days)	ml. of 0.1 normal acid equiv. to nitrogen of 5 ml. of skimmilk serum								
		Total N.	Nitrogen fractionated into sol. and insol. portions with							* Amino N.
			Trichloracetic acid		Ethyl alcohol		Phosphotungstic acid			
			Sol.	Insol	Sol.	Insol.	Sol.	Insol.		
Held at 37°C.										
39	2	2.9	2.9	0.2	1.3	1.9	1.2	2.0		1.76
170	2	8.0	7.5	1.2	4.5	3.7	1.4	6.3		1.92
98	2	2.6	2.4	0.5	1.4	1.4	1.0	1.7		1.14
110	2	2.7	1.8	0.6	1.6	1.3	1.3	1.5		1.85
Control	2	2.6	2.6	0.3	1.8	0.6	1.2	1.5		0.92
39	4	3.0	2.6	0.4	1.3	1.9	1.4	2.0		1.88
170	4	11.2	9.9	1.0	6.8	4.8	2.4	8.8		3.35
98	4	3.5	2.8	0.3	1.2	2.5	1.4	1.9		1.42
110	4	3.0	2.5	0.5	1.2	2.1	1.7	1.8		1.89
Control	4	2.7	2.5	0.4	1.3	1.3	1.0	1.9		0.64
39	7	3.8	3.4	0.6	1.6	1.9	1.2	2.5		1.98
170	7	11.5	11.0	0.5	6.2	4.8	2.2	8.7		3.59
98	7	7.4	6.6	0.5	4.3	2.8	2.6	4.7		3.14
110	7	4.1	3.3	0.6	2.5	1.3	1.9	2.1		1.92
Control	7	2.3	2.4	0.3	1.3	1.4	1.1	1.5		0.71

Table 3. (continued)

Culture no.	Incubation time (days)	ml. of 0.1 normal acid equiv. to nitrogen of 5 ml. of skimmilk serum							* Amino N.
		Total N.	Nitrogen fractionated into sol. and insol. portions with						
			Trichloracetic acid		Ethyl alcohol		Phosphotungstic acid		
			Sol.	Insol.	Sol.	Insol.	Sol.	Insol.	
		Held at 21°C.							
39		3.3	2.5	0.6	1.7	2.0	1.5	1.9	1.78
170		5.7	5.7	0.4	2.5	2.9	1.0	4.2	2.00
98	2	2.4	2.1	0.4	1.2	1.4	1.0	1.7	1.50
110		2.5	2.4	0.2	1.4	1.4	1.4	1.5	1.50
Control		2.7	2.6	0.3	1.5	1.2	1.2	1.5	0.85
39		4.8	4.5	0.4	2.0	3.0	1.5	3.5	2.14
170		8.4	8.1	0.5	4.4	4.1	1.1	7.5	2.38
98	4	2.7	2.4	0.3	1.6	1.3	1.5	1.5	1.57
110		3.0	2.7	0.5	1.7	1.3	1.4	2.0	1.64
Control		2.8	2.8	0.4	1.5	1.2	1.4	1.5	0.78
39		5.7	5.5	0.4	2.9	3.0	1.2	4.7	2.32
170		11.5	9.8	1.4	6.1	5.5	2.1	9.8	3.57
98	7	2.8	2.5	0.5	1.5	1.5	0.8	1.6	1.59
110		9.4	9.5	0.1	6.2	6.1	5.0	6.1	2.98
Control		2.5	2.5	0.1	1.2	1.5	0.9	1.5	0.71

* Calculated from milligrams of amino nitrogen as obtained by the Van Slyke gasometric method.

more breakdown at the higher temperature, while cultures 39 and 110 produced more breakdown at the lower one.

The distribution of nitrogen in the various fractions was much the same with the different organisms and with the two incubation temperatures. The fractions soluble in trichloracetic acid were relatively large and increased with continued incubation, while the values for the fraction insoluble in trichloracetic acid, as would be expected, were regularly low even after 7 days incubation. The amounts of nitrogen soluble and insoluble in ethyl alcohol were more or less the same, and each of the amounts generally increased with continued incubation. When phosphotungstic acid was used as the precipitant, most of the nitrogen was contained in the insoluble fractions; the soluble fractions remained about the same during incubation, whereas the insoluble fractions generally increased.

The amounts of amino nitrogen increased with continued incubation at both temperatures. There was a very general relationship between the values obtained for total nitrogen and those obtained for amino nitrogen; when the values for total nitrogen were high, those for amino nitrogen were also high.

General Action of Proteolytic Micrococci on Unsalted Butter

The general action of 58 cultures of proteolytic micrococci on butter was studied by churning small portions of sterile cream inoculated with test organisms; this was done by agitating in a motor driven shaking machine. The cream was prepared by placing 500 ml. portions in 1-quart glass top fruit jars and heating in an autoclave at 12 pounds pressure for 30 minutes on 2 consecutive days. A culture of each test organism was prepared by inoculating 100 ml. of sterile skimmilk and incubating at 37°C. for 48 hours. At the time of churning all of a milk culture was added to a jar of cream. The relatively large inoculation was used to insure many test organisms in the butter since washing of the butter would remove many of them. From four to six jars of cream constituted a series, one of the jars being left uninoculated as a control.

The butter was washed three times with sterile water and worked after each washing; the working after the last washing was intended to give a good distribution of the moisture droplets. All the equipment used in handling the butter was sterile. The butter was left unsalted so that the conditions for growth would be more satisfactory than if salt were added. Each churning was then divided and held in sterile petri dishes at about 4°C. and 20°C. At several intervals during the holding periods of from 1 to

8 weeks the butter was scored and criticized.*

The control butter in each series was treated in the same manner as the butter containing the test organisms. In general, the control butter kept reasonably well, although occasionally it developed mold growth or an off flavor, presumably as a result of a contamination. When this occurred the entire series of churnings was discarded. The uniformity of scores on the control butter and the fresh butter made from cream inoculated with the test organisms can be explained by the fact that the cream used in all the churnings came from the same source and that the cream was sterilized rather than pasteurized. The heat treatment of the cream caused an oily flavor in the butter, but this was considered preferable to the use of pasteurized cream which might contain considerable numbers of organisms in addition to the test organism.

Action of orange micrococci

Fourteen cultures of the orange micrococci were studied from the standpoint of their action on butter. The data on one of these cultures, which was considered representative, are given in Table 4. Culture No. 49 brought about relatively rapid changes in the flavor of butter. After 4 to 7 weeks at 4°C. or after 1 to 2 weeks at 20°C. definite flavor defects

* Scores and criticisms were given by Prof. F. H. Herzer of the Dairy Department, Mississippi State College.

Table 4.

General action of orange culture No. 49 on butter

Churning no.	Temp. held (°C.)	Period for development of definite defect (weeks)	Score and general criticism on butter		Decrease in score
			At churning	After storage	
1	4	6	90 oily, flat	88 tallowy sl* rancid	2
	20	2	90 oily, flat	85 rancid	5
2	4	7	90 oily, flat	86.5 rancid	3.5
	20	2	90 oily, flat	87.5 sl. rancid	2.5
3	4	6	90 oily, flat	87 sl. rancid	3
	20	1	90 oily, flat	88 cheesy	2
4	4	4	90 oily, flat	87.5 sl. rancid	2.5
	20	1	90 oily, flat	87.5 sl. rancid	2.5
5	4	6	90 oily, flat	88 sl. rancid	2
	20	1	90 oily, flat	88 sl. rancid	2

* Sl. = slightly

were apparent. A rancid flavor generally developed in the samples although in churning No. 5 a distinct cheesy flavor was evident at 20°C. The scores on the butter decreased from 2 to 5 points between the time of churning and the end of the holding periods.

Table 5 summarizes the data on the cultures of orange micrococci. The results show that relatively rapid changes were brought about in the flavor of butter by the strains of orange micrococci employed. When the samples were held at 4°C., rancid, cheesy or pungent flavors commonly developed after 4 to 7 weeks, while with samples held at 20°C. the same general defects were conspicuous after 1 to 5 weeks. A few of the cultures failed to produce a rancid or putrid defect at 4°C., which presumably was due to a slow growth at relatively low temperature. Decreases in the scores of samples during the holding varied from 1.5 to 5 points. The decreases in score were frequently greater with the relatively short holding at 20°C. than with the longer holding at 4°C.

As a group the orange proteolytic micrococci are lipolytic so that action on both the protein and fat of butter would be expected if the holding temperature and other conditions permitted growth. Various organisms which attack protein and fat give both cheesy and rancid flavors in butter, although commonly a rancid flavor tends to predominate.

Table 5.

General action of orange proteolytic micrococci on butter

Culture no.	No. of churnings	Temp. held (°C.)	Period for development of definite defect (weeks)	Average score and general criticism on butter			Decrease in score
				At churning	After storage		
7	3	4	5	90 oily, flat	86	rancid cheesy	4
		20	5	90 oily, flat	86	rancid	4
8	1	4	5	90 oily, flat	87	rancid	3
		20	1	90 oily, flat	85.5	rancid bitter	4.5
10	3	4	6	90 oily, flat	88	oily cheesy	2
		20	3	90 oily, flat	85	cheesy rancid	5
12	1	4	4	90 oily, flat	86	oily bitter	4
		20	1	90 oily, flat	85	rancid pungent	5
13	4	4	6	90 oily, flat	86	rancid	4
		20	1	90 oily, flat	85	rancid pungent	5
18	2	4	7	90 oily, flat	88	bitter tallowy	2
		20	2	90 oily, flat	88	sl.* rancid	2
30	5	4	6	90 oily, flat	88	sl. rancid	2
		20	3	90 oily, flat	85	rancid pungent	5

Table 5. (continued)

Culture no.	No. of churnings	Temp. held (°C.)	Period for development of definite defect (weeks)	Average score and general criticism on butter		Decrease in score
				At churning	After storage	
31	2	4	4	90 oily, flat	86 sl. rancid	2
		20	2	90 oily, flat	85 rancid putrid	5
33	5	4	6	90 oily, flat	87 sl. rancid	3
		20	1	90 oily, flat	88 sl. rancid	2
34	2	4	8	90 oily, flat	88.5 oily	1.5
		20	1	90 oily, flat	86 rancid	4
36	6	4	6	90 oily, flat	87 sl. rancid	3
		20	2	90 oily, flat	86 rancid	4
39	7	4	6	90 oily, flat	85.5 oily storage	1.5
		20	2	90 oily, flat	85 rancid pungent	5
43	2	4	8	90 oily, flat	88.5 oily storage	1.5
		20	2	90 oily, flat	86 putrid	4
49	5	4	6	90 oily, flat	88 tallowy sl.rancid	2
		20	2	90 oily, flat	86 rancid	4

* Sl. = Slightly

Action of yellow micrococci

The action of the yellow micrococci on butter was studied with 11 cultures. The data on one representative culture are given in Table 6. Culture No. 63 produced a variety of changes in the flavor of butter. After 4 to 8 weeks at 4°C. or 2 to 5 weeks at 20°C., flavor defects were conspicuous and included musty, bitter, cheesy, tallowy and rancid flavors. The score on the butter decreased from 0.5 to 3.0 points between the time of churning and the end of the holding.

Table 7 summarizes the results obtained with the 11 yellow cultures. The data show that changes were produced in the flavor of butter by most of the yellow strains studied although the changes occurred relatively slowly. When samples were held at 4°C., the most common defects were cheesy, musty or bitter flavors which developed after 4 to 8 weeks. With the samples held at 20°C., similar defects were detected in from 2 to 5 weeks. Decreases in the scores of the samples held at both temperatures varied from 0.5 to 3.5 points; in some cases the decreases in score were considerably greater at 20°C. than at 4°C. In general, members of the yellow group were relatively slow in producing definite flavor defects in butter compared to members of the orange group (Table 5). Although in three churnings a slight rancid flavor was detected in the butter, the group as a whole is nonlipolytic. The chief defect in butter caused by members of this group was a cheesy flavor which was probably due to the protein breakdown.

Table 6.

General action of yellow culture No. 65 on butter

Churning no.	Temp. held (°C.)	Period for development of definite defect (weeks)	Score and general criticism on butter: At churning	Score and general criticism on butter: After storage	Decrease in score
1	4	5	90 oily, flat	87.5 bitter musty	2.5
	20	3	90 oily, flat	87.5 oily musty	2.5
2	4	8	90 oily, flat	89 sl.* tallowy	1
	20	5	90 oily, flat	87 sl. rancid	3
3	4	5	90 oily, flat	88 bitter	2
	20	4	90 oily, flat	87.5 cheesy	2.5
4	4	4	90 oily, flat	89.5 storage	0.5
	20	3	90 oily, flat	87 musty	3
5	4	7	90 oily, flat	89.5 oily	0.5
	20	4	90 oily, flat	88 oily	2

Sl.= Slightly

Table 7.

General action of yellow proteolytic micrococci on butter

Culture no.	No. of churnings	Temp. held (°C.)	Period for development of definite defect (weeks)	Average score and general criticism on butter		Decrease in score
				At churning	After storage	
65	5	4	8	90 oily, flat	88.5 oily	1.5
		20	4	90 oily, flat	86.5 cheesy musty	3.5
67	4	4	6	90 oily	88 oily cheesy	2
		20	4	90 oily	88 oily musty	2
74	2	4	4	90 oily, flat	88.5 sl.* rancid	1.5
		20	2	90 oily, flat	86.5 rancid	3.5
85	6	4	7	90 oily, flat	88 sl. rancid	2
		20	5	90 oily, flat	87 cheesy musty	3
97	4	4	7	90 oily, flat	87.5 cheesy	2.5
		20	4	90 oily, flat	88 sl. cheesy	2
98	5	4	6	90 oily, flat	88 musty	2
		20	3	90 oily, flat	88 sl. cheesy	2
99	2	4	7	90 oily	88.5 sl. musty	1.5
		20	3	90 oily	88 cheesy	2

* Sl. = Slightly

Table 7 (continued)

Culture no.	No. of churnings	Temp. held (°C.)	Period for development of definite defect (weeks)	Average score and general criticism on butter: At churning		After storage		Decrease in score
110	1	4	6	90	oily, flat	88.5	storage	1.5
		20	3	90	oily, flat	87.5	cheesy musty	3.5
116	1	4	4	90	oily, flat	89	oily	1
		20	4	90	oily, flat	87	cheesy	3
154	1	4	5	90	grass flavor	89.5	oily	0.5
		20	5	90	grass flavor	87	bitter	3
157	1	4	7	90	oily	89	oily	1
		20	2	90	oily	88.5	oily sl.* rancid	1.5

*Sl. = slightly

Action of white micrococci

Fourteen cultures of the white micrococci were studied from the standpoint of their action on butter. The data on one of these cultures, which was considered representative, are given in Table 8. Butter containing culture No. 175 commonly developed a cheesy flavor after 4 to 7 weeks at 4°C. or after 1 to 3 weeks at 20°C. The scorings were rather uniform and the decreases in score during holding ranged from 1 to 3 points.

Table 9 summarizes the data obtained with the 14 white cultures. The results indicate that the organisms brought about comparatively slow changes in the flavor of butter at 4°C., while relatively rapid changes were noted at 20°C. At 4°C., oily or bitter flavors developed after 4 to 6 weeks, whereas at 20°C. most of the samples developed a cheesy flavor in 1 to 4 weeks. Decreases in the scores of samples during holding varied from 0.5 to 4 points. In general, the defects caused by this group were not as pronounced as those brought about by the orange cultures (Table 5.), and were more comparable with those caused by the yellow cultures (Table 7.).

The principal defect in butter caused by the white proteolytic micrococci was a cheesy or bitter flavor which presumably was due to protein decomposition in the butter. No lipolytic cultures were studied in this group and in only a few cases was the butter found to have a slightly rancid flavor.

Table 8.

General action of white culture No. 175 on butter

Churning no.	Temp. held (°C.)	Period for development of definite defect (weeks)	Score and general criticism on butter		Decrease in score
			At churning	After storage	
1	4	4	90 oily, flat	88.5 musty	1.5
	20	1	90 oily, flat	88 bitter	2
2	4	4	90 oily, flat	88 oily bitter	2
	20	3	90 oily, flat	88 cheesy	2
3	4	7	90 oily, flat	88.5 sl.* tallowy	1.5
	20	2	90 oily, flat	88.5 cheesy	1.5
4	4	5	90 oily, flat	88 storage	2
	20	3	90 oily, flat	88 cheesy	2
5	4	6	90 oily, flat	88.5 cheesy	1.5
	20	3	90 oily, flat	87.5 oily sl. cheesy	2.5
6	4	6	90 oily, flat	89 oily	1
	20	2	90 oily, flat	87 oily cheesy	3

* Sl. = Slightly

Table 9.

General action of white proteolytic micrococci on butter

Culture no.	No. of churnings	Temp. held (°C.)	Period for the development of definite defect (weeks)	Average score and general criticism on butter		Decrease in score
				At churning	After storage	
175	6	4	6	90 oily, flat	88 oily	2
		20	2	90 oily, flat	87.5 oily cheesy	2.5
200	2	4	6	90 oily, flat	88 oily storage	1.5
		20	2	90 oily, flat	88.5 oily	1
250	5	4	6	90 oily, flat	89 oily	1
		20	2	90 oily, flat	88.5 oily bitter	1.5
260	3	4	5	90 oily, flat	89 sl.* bitter	1
		20	4	90 oily, flat	88 cheesy	2
274	3	4	4	90 oily, flat	89 oily	1
		20	2	90 oily, flat	87 sl. rancid	3
286	2	4	4	90 oily, flat	89 oily	1
		20	2	90 oily, flat	87.5 cheesy	2.5
289	1	4	4	90 oily, flat	88.5 oily bitter	1.5
		20	1	90 oily, flat	88 oily bitter	2

* SL. = Slightly

Table 9. (continued)

Culture no.	No. of churnings	Temp. held (°C.)	Period for the development of definite defect (weeks)	Average score and general criticism on butter: At churning	Average score and general criticism on butter: After storage	Decrease in score
310	1	4	4	90 oily, flat	89.5 oily	0.5
		20	3	90 oily, flat	87 flat sl* cheesy	3
315	4	4	6	90 oily, flat	87.5 cheesy	2.5
		20	2	90 oily, flat	87 oily bitter	3
317	2	4	4	90 oily, flat	88 oily storage	2
		20	1	90 oily, flat	87.5 cheesy	2.5
325	4	4	5	90 oily, flat	88 oily cheesy	2
		20	3	90 oily, flat	87.5 sl. rancid	2.5
328	2	4	4	90 oily, flat	87 musty bitter	3
		20	2	90 oily, flat	86 musty cheesy	4
335	1	4	6	90 oily, flat	89.5 oily	0.5
		20	2	90 oily, flat	87.5 cheesy	2.5
412	2	4	5	90 oily, flat	88 oily sl. rancid	2
		20	3	90 oily, flat	87.5 tainted	2.5

* Sl.= slightly

Species of Proteolytic Micrococci

An attempt was made to identify, on a species basis, the 426 cultures of proteolytic micrococci isolated from various dairy products. All the organisms were put in the genus Micrococcus, although some of them are included in the genus Staphylococcus by Bergey (3). This arrangement is in agreement with Hucker (25) who has considered the advisability of placing the organisms that have been variously described as micrococci, staphylococci and rhodococci in the single genus Micrococcus. It meets the problem of classifying the organisms of this general group that are so widely distributed in dairy products better than a division into two or more genera. The characters given for the various species by Bergey (3) were used in the identification.

Orange Group

Forty-eight of the 53 cultures of orange micrococci were identified as Micrococcus aureus; of these cultures, 39 were isolated from milk, 6 from butter, 1 from cheese and 2 from ice cream.

Five of the orange cultures could not be identified on the basis of the species described by Bergey (3). Some investigators would probably consider them M. aureus, but they differ from M. aureus in so many respects that a distinction seems advisable. The 5 cultures appear to repre-

sent three species which are designated Micrococcus v, Micrococcus w and Micrococcus x in Table 2.

Micrococcus v

Two strains of this organism were isolated from milk. They differ from M. aureus in their action on litmus milk, nitrate reduction and carbohydrate fermentation and from Micrococcus w and Micrococcus x in action on litmus milk, ammonia nitrogen assimilation and carbohydrate fermentation.

Micrococcus w

One strain of this culture was isolated from milk. It differs from M. aureus in nitrate reduction, diastatic action and its ability to ferment dextrine, maltose, salicin and sorbitol, and from Micrococcus x in action on litmus milk, nitrate reduction, ammonia nitrogen assimilation and carbohydrate fermentation.

Micrococcus x

Two strains of this culture were isolated from milk. They differ from M. aureus in action on litmus milk, ammonia nitrogen assimilation, hemolysis and ability to ferment maltose, salicin and sorbitol.

Yellow Group

Forty-one cultures of the 115 yellow micrococci were

identified as Micrococcus flavus; of these, 35 were isolated from milk, 2 from butter and 4 from ice cream. Eighteen cultures were identified as Micrococcus flavescens; 12 of these were isolated from milk and 6 from cheese. Twenty-four cultures were identified as Micrococcus citreus; of these, 6 were obtained from milk, 4 from butter, 2 from cheese and 12 from ice cream. Thirty-two cultures were identified as Micrococcus conglomeratus; all of these were isolated from milk.

White Group

Fifty-six of the 258 white micrococci were identified as Micrococcus albus; 37 of these were isolated from milk, 10 from butter, 4 from cheese and 5 from ice cream. Seventeen cultures were identified as Micrococcus cremoris-viscosi; all of these were isolated from milk. Eighty-eight cultures were identified as Micrococcus freudenreichii; of these 73 were isolated from milk, 8 from butter and 7 from cheese. Eighty-four cultures were identified as Micrococcus caseolyticus; 68 of these were obtained from milk, 8 from butter and 8 from ice cream. Eleven cultures were identified as Micrococcus epidermidis; all of these were isolated from milk.

Eight of the white cultures could not be identified on the basis of the species described by Bergey (3). These cultures appear to represent two species which are designated

Micrococcus y and Micrococcus z in Table 2.

Micrococcus y

Six cultures were obtained from milk. They differ from the identified species of micrococci in their action on litmus milk and the fermentation of certain carbohydrates and from Micrococcus z in their ability to ferment certain carbohydrates.

Micrococcus z

Two cultures were isolated from milk. They differ from the identified species of micrococci in their action on milk and the fermentation of certain carbohydrates.

Key to the Identification of Proteolytic Micrococci

A. Orange pigment produced

1. Nitrates reduced

a. Acid in litmus milk without coagulation
Micrococcus x

aa. Acid and coagulation in litmus milk
Micrococcus aureus

2. Nitrates not reduced

a. Acid and coagulation in litmus milk
Micrococcus w

aa. No change in litmus milk
Micrococcus v

B. Yellow pigment produced

1. Nitrates reduced

a. Acid reaction in litmus milk
Micrococcus citreus
aa. No change in litmus milk
Micrococcus conglomeratus

2. Nitrates not reduced

a. Acid reaction in litmus milk
Micrococcus flavus
aa. No change in litmus milk
Micrococcus flavescens

C. White pigment produced

1. Nitrates reduced

a. Acid reaction in litmus milk
b. Ferment sucrose
c. Ferment glycerol
Micrococcus caseolyticus
cc. Do not ferment glycerol
Micrococcus albus
bb. Do not ferment sucrose
Micrococcus cremoris-viscosi
aa. No change in litmus milk
Micrococcus epidermidis

2. Nitrates not reduced

a. Alkaline reaction in litmus milk
b. Ferment dextrose
Micrococcus y
bb. Do not ferment dextrose
Micrococcus z
aa. Acid reaction in litmus milk
Micrococcus freudenreichii

DISCUSSION OF RESULTS

The relatively large numbers of proteolytic micrococci encountered in detailed studies on the distribution of these organisms in various dairy products is in agreement with the observations of investigators who had repeatedly obtained proteolytic micrococci in attempts to isolate other types of organisms that attack protein of milk. They also suggest that the organisms may be of importance from the standpoint of various changes in dairy products. Although many investigators have considered the micrococci a more or less inert group, producing changes rather slowly, various cultures were obtained which brought about comparatively rapid changes in milk and unsalted butter. Extensive proteolysis was produced in milk by certain of the organisms in relatively short periods and butter made from cream inoculated with the organisms showed definite flavor defects rather early.

The defects produced in butter indicated both proteolysis and lipolysis by the organisms. The fact that some of the organisms studied were isolated from cheese suggests they may be important in the cheese ripening process, since proteolysis and lipolysis appear to be of significance in the flavor development of various cheeses.

In detecting proteolysis by micrococci, it appears that culturing on milk agar plates is of greater value than cultur-

ing in litmus milk, since certain cultures which gave very evident proteolysis on milk agar did not produce changes in the appearance of litmus milk justifying the conclusion that the organisms were proteolytic. With some cultures other types of changes in milk, such as acid development, tended to obscure proteolysis and possibly to retard it. Some of the proteolytic micrococci did not liquefy gelatine. This deviation from a relationship that might be expected is in agreement with observations reported for organisms belonging to various genera.

The relatively minor effect of various factors on the color production of the cultures on agar emphasizes the value of chromogenesis in the classification of the organisms.

The viability of the cultures on agar and in milk parallels the general resistance of the micrococci to various unfavorable conditions that has been noted in investigations on these organisms. It indicates that contamination of dairy products with material containing these organisms may be greatly delayed without the organisms being destroyed. The general viability greatly simplifies the carrying of cultures.

The cultures studied were all placed in the genus Micrococcus since, in general, the differences among them did not appear to justify division into two or more genera. An important point in this connection was the failure of representative cultures to kill guinea pigs with which they were inoculated, since the genus Staphylococcus is characterized

by Bergey (3) as "usually parasitic". There is precedent for including all the cultures in the genus Micrococcus in Hucker's treatment (25) of this general group.

While most of the characters that have been used by various investigators in classifying the organisms of the genus Micrococcus are of importance, it appears that more attention should be given to the changes produced in litmus milk and that hydrolysis of fat should be included as a differentiating character. The changes produced in litmus milk permit considerable differentiation of the organisms. The detection of fat hydrolysis is now relatively simple because of the development of methods that are easily applied.

The cultures that could not be identified by the descriptions of organisms listed by Bergey (3) were not given species names for several reasons. There has been a definite attempt, especially by Hucker (24), to reduce the number of recognized species of micrococci because the large number of species described has resulted in much confusion. It appears that new species should only be suggested when the evidence is complete and when a considerable number of cultures is available for study, particularly from the standpoint of the variations to be expected within the genus. If additional isolations of the cultures are made, species names must eventually be proposed.

SUMMARY AND CONCLUSIONS

By plating on milk agar and incubating at 37°C. for 48 to 72 hours, proteolytic micrococci were readily isolated from many samples of milk, butter, cheese and ice cream. They were obtained from 340 of 500 samples of milk, from 36 of 42 sample of butter, from 20 of 36 samples of cheese (cheddar) and from 30 of 46 samples of ice cream.

On the basis of chromogenesis, the proteolytic micrococci were readily divided into four groups--red, orange, yellow and white (non-pigmented). The red micrococci were not studied in detail because of the small numbers encountered.

Studies on the influence of various factors on chromogenesis indicated:

(a) That when grown in an atmosphere of carbon dioxide, the general pigment production on agar was not significantly different than when grown in air, although with a few of the orange cultures the pigment was lighter in color.

(b) That when grown on potato, some of the orange organisms produced a deeper color than when grown on agar.

(c) That the pH value of the medium had an effect on the pigment production, a value of 7.0 being more favorable than values of 6.6 or 7.6, although pigment production was definite at all three values.

(d) That holding organisms in sterile water for 6 days before

inoculating on agar did not influence pigment production.

(e) That subjecting cultures to 55°C. for 10 minutes before inoculating on agar had little or no effect on pigment production.

(f) That more highly colored pigment was produced at 21°C. than at 37°C. with the orange cultures but not with the yellow cultures.

(g) That agar cultures held in darkness for 10 days were not as deeply pigmented as when held in a sun lighted room.

Milk cultures of the proteolytic micrococci were viable for 3 to 4 months and agar slant cultures for 10 to 12 months at room temperature.

When litmus milk was inoculated with the various organisms, four general types of changes were produced while a few cultures produced no noticeable change.

The titratable acidity produced in milk by 20 representative cultures varied from 0.12 to 0.66 per cent, calculated as lactic acid. Organisms of the orange and white groups generally gave higher values than those of the yellow group.

All of the cultures studied grew well on beef infusion agar slants and in beef broth.

A majority of the cultures studied reduced nitrates.

In general, the organisms did not assimilate amino

nitrogen, although ammonia nitrogen was assimilated by many cultures of the orange and yellow groups but by none of the white group. All cultures were capable of producing ammonia from peptone, whereas none produced ammonia from nitrates.

All cultures except a few of the white group liquefied gelatine.

Cultures of the yellow group generally produced larger amounts of carbon dioxide than cultures of the orange or white groups.

Hemolysis was shown by some cultures of the orange and yellow groups but by none of the white group.

Lipolysis was limited to organisms of the orange group. None of the yellow or white organisms studied were lipolytic, according to the methods used.

Diastatic action was exhibited by 1 culture of the orange group and by 96 of the yellow group. None of the white cultures produced diastase.

In general, most of the cultures studied fermented dextrose, galactose, lactose, levulose and maltose. Dextrine, glycerol, mannitol, salicin, sorbitol, sucrose and xylose were fermented by some cultures.

Arabinose and raffinose were not fermented.

In broth cultures all of the organisms were destroyed at temperatures ranging from 55° to 65°C. for 15 minutes.

Appreciable amounts of acetylmethylcarbinol plus diacetyl or volatile acid were not produced by the organisms.

Tests for hydrogen sulphide and indol were regularly negative.

Thirty representative cultures were found to be non-pathogenic when inoculated into guinea pigs intraperitoneally.

None of the organisms studied produced spores, and all were apparently non-motile.

Protein breakdown in skimmilk by four representative cultures of proteolytic micrococci was studied in detail. When grown in milk at 37° or 21°C. for 2 to 7 days, the organisms increased the amount of nitrogen in the serum, although the extent of proteolysis varied considerably with the cultures. In general, the fractions of nitrogen soluble in trichloracetic acid, soluble and insoluble in ethyl alcohol or insoluble in phosphotungstic acid increased during the incubation, whereas the fractions insoluble in trichloracetic acid or soluble in phosphotungstic acid remained about the same. The amounts of amino nitrogen increased in the serum during the holding.

The general action of 38 cultures of proteolytic micrococci on unsalted butter was studied by churning small portions of sterile cream inoculated with relatively large numbers of the organisms, and then examining the butter after various periods at 4° and 20°C. Definite defects were produced in many samples of butter after 4 to 8 weeks at 4°C. or 1 to 5 weeks at 20°C. Certain cultures of the orange group produced rancid, cheesy and pungent flavors, certain of the yellow group produced cheesy, musty or bitter flavors, while certain of the white group brought about oily or bitter flavors. In

general, members of the orange group changed the flavor of butter more rapidly and more extensively than members of the yellow or white groups.

An attempt was made to identify, on a species basis, the 426 cultures of proteolytic micrococci. The organisms were separated into 15 species of the genus Micrococcus on the basis of chromogenesis, action on nitrates, action on milk and the fermentation of various carbohydrates. Ten species were identified from descriptions given by other investigators, while 5 species were unidentified. A key to the identification of the 15 species of proteolytic micrococci is given.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. B. W. Hammer for his wise counsel in planning and directing the work herein reported and in the preparation of the manuscript; to Dr. C. B. Lane for his kind assistance on nitrogen work, and to Prof. F. H. Herzer for his assistance in scoring butter.

LITERATURE CITED

1. Allen, P. W. A simple method for the classification of bacteria as to diastase production. Jour. Bact. 3: 15-17. 1918.

2. Bailey, S. F. and Lacy, G. R. A modification of the Kligler lead acetate medium. Jour. Bact. 13: 183-189. 1927.

3. Bergey, D. H. Manual of determinative bacteriology. 4th Ed. Williams and Wilkins Co. Baltimore. 1934.

4. Breed, Alice F. Micrococci present in the normal cow's udder. N. Y. (Geneva) Agr. Exp. Sta., Tech. Bul. 152. 1928.

5. Buchanan, R. E. Studies on the nomenclature and classification of the bacteria IV. Subgroups and genera of the Coccaceae. Jour. Bact. 2: 603-617. 1917.

6. Burkey, L. A. Methods for determining the persistence of certain important bacteria in Swiss cheese. Jour. Bact. 21: 40-41. 1931.

7. Chester, F. D. A manual of determinative bacteriology. Macmillan Co. New York. 1901.

8. Cohn, F. Untersuchungen über Bakterien. Beitr. z. Biol. d. Pflanzen, 1 (Heft 2): 127-224. 1872. Original not seen; cited by G. J. Hucker, 22, p.7.

9. Cohn, F. Organismen in der Pockenlymphe. Virchow's Arch. f. Path. Anat., 55: 229-258. 1872 Original not seen; cited by G. J. Hucker, 22, p.7.

10. Cohn, F. Untersuchungen über Bakterien. II. Beitr. z. Biol. d. Pflanzen 1: (Heft 3): 141-207. 1875. Original not seen; cited by G. J. Hucker, 22, p.7.

11. Copeland, Lynn, and Olson, T. M. The bacterial flora of normal cow's udder. U. D. Agr. Exp. Sta., Bul. 218. 1926.

12. Dudgeon, L. S. The differentiation of the Staphylococci. Jour. Path. and Bact. 12: 242-257. 1908.

13. Eldredge, E. E. and Rogers, L. A. The bacteriology of cheese of the Emmental type. Centbl. Bakt. 2. Abt. 40: 5-21. 1914.

14. Evans, Alice C. The bacteria of milk freshly drawn from normal udders. Jour. Inf. Dis. 18: 437-476. 1916.

15. Frazier, W. C. and Rupp, Phillip. Studies on the proteolytic bacteria of milk. II. Action of the proteolytic bacteria of milk on milk. Jour. Bact. 16: 65-78. 1928.

16. Freudenreich, E. von. Beitrag zur Kenntnis der Ursachen des bitteren Käses und der bitteren Milch. Landw. Jahrb. d. Schweiz, 8: 135-148. 1894. (Original not seen) Cited in Jordan, E. O. and Falk, I. S. A newer knowledge of bacteriology and immunology. p. 879; University Chicago Press. 1928.

17. Gorini. C. The acidoproteolytes in the pasteurization of milk for the manufacture of soft cheeses of "Bel Paese" type. Proc. 10th World's Dairy Congress, Rome 10: Sec. 3: 7-15. 1934.

18. Hallier, E. Bewis, dass der Micrococcus der Infektions krankheiten keimfähig und von höheren Pilzformen abhängig ist, und Widerlegung der leichsinnigen Angriffe des Herrn Collegen Bary zu Halle. Leitschrift für Parasitenkunde. 2: 1-20. 1870.

19. Hammer, B. W. and Bailey, D. E. The volatile acid production of starters and of organisms isolated from them. Iowa Agr. Exp. Sta., Res. Bul. 55. 1919.

20. Hammer, B. W. and Collins, M. A. The numbers of lipolytic bacteria in various dairy products, as determined with nileblue sulphate. Iowa Agr. Exp. Sta., Res. Bul. 169. 1934.

21. Harding, H. A. and Wilson, K. J. A study of the udder flora of cows. N. Y. (Geneva) Agr. Exp. Sta., Tech. Bul. 27. 1913.

22. Hucker, G. J. Studies on the Coccaceae. I. Previous taxonomic studies concerning the genera of the Coccaceae. N. Y. (Geneva) Agr. Exp. Sta., Tech. Bul. 99. 1924.

23. Hucker, G. J. The types of bacteria found in commercial cheddar cheese. N. Y. (Geneva) Agr. Exp. Sta., Tech. Bul. 90. 1923.

24. Hucker, G. J. Studies on the Coccaceae II. A study of the general characters of the micrococci. N. Y. (Geneva) Agr. Exp. Sta., Tech. Bul. 100. 1924.

25. Hucker, G. J. Studies on the Coccaceae IV. Classification of the genus Micrococcus Cohn. N. Y. (Geneva) Agr. Exp. Sta., Tech. Bul. 102. 1924.

26. Kligler, I. J. A systematic study of the Coccaceae in the collection of the Museum of Natural History. Jour. Inf. Dis. 12: 452-453. 1913.

27. Knowles, N. R. A study of the bacterial flora of foremilk and of rennet extract with special reference to acid-proteolytic types. Jour. Dairy Res. 7: 63-74. 1936.

28. Lane, C. B. and Hammer, B. W. Bacteriology of cheese. I. Effect of pasteurizing the milk on the nitrogenous decomposition in cheddar cheese. Iowa Agr. Exp. Sta., Res. Bul. 185. 1935.

29. Long, H. F. A study of some lipolytic microorganisms isolated from dairy products. Unpublished Thesis, Iowa State College Library, Ames. 1936.

30. Michaelian, M. B. and Hammer, B. W. Studies on acetylmethylcarbinol and diacetyl in dairy products. Iowa Agr. Exp. Sta., Res. Bul. 179. 1935.

31. Migula, W. System der Bakterien. G. Fischer, Jena. 1897.

32. Orla-Jensen, S. Dairy Bacteriology. P. Blakiston's Son and Co. Philadelphia. 1921.

33. Society of American Bacteriologists, Committee on Bacteriological Technique Manual of methods for pure culture study of bacteria. 2: Leaflet 5. 1934.

34. Van Slyke, D. D. A method for quantitative determination of aliphatic amino groups. Jour. Bio. Chem. 9: 185. 1911.

35. Winslow, C. E.-A. and Winslow, A. R. The systematic relationships of the Coccaceae. John Wiley and Sons, New York. 1908.

36. Winslow, C. E.-A., Rothberg, W., and Parsons, E. I. Notes on the classification of the white and orange staphylococci. Jour. Bact. 5: 145-167. 1920.

37. Winslow, C. E.-A., Broadhurst, Jean, Buchanan, R. E., Krumwiede, Charles, Rogers, L. A., and Smith, G. H. The families and genera of the bacteria. Jour. Bact. 5: 191-229. 1920.